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A lipid dependence in the formation of twin ion channels

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Abstract

A gramicidin A derivative with a polyether linkage between both ethanolamine termini was synthesized and its ion channel properties were studied. The compound showed a duplication in the state of conductance for alkali cations in thick DOPC bilayer membranes, which is interpreted as the occurrence of twin-channels. In thinner DMPC membranes mono-channels were dominant. The influence of hydrophobic coupling on the mono channel/twin channel equilibrium is discussed.

Keywords: Gramicidin A; Ion channel; Bilayer membrane; Twin-channel; Hydrophobic coupling

Although there is high lateral mobility in membranes, the lateral organization of integral membrane proteins can be of functional significance [1]. Protein clustering was observed, e.g., for ion channels [2]. Ion channels are suitable objects to study cluster effects, because their functional analysis can be performed easily by current measurements even on the single molecule level [3].

Gramicidin A (**gA**) is a prominent lead structure for ion-channel studies and ion-channel engineering [4,5]. **gA**, a peptidic antibiotic with 15 amino acids in length, is produced by *Bacillus brevis*, and consists of alternating L- and D-amino acids (Fig. 1A).

In membrane-like environments **gA** forms a head-to-head dimer of two right-handed single-stranded β-helices [6]. This structure with 6.3 residues per turn is the accepted ion-channel structure [7–9]. The channel-active **gA** dimer is via dissociation in equilibrium with the channel-inactive monomer. Compound **1** represents a **gA** derivative with a N-Boc protected aminodiethyleneglycol chain (in red) covalently connected to the aminoethanol endgroup. In a phospholipid bilayer, two molecules of **1** can associate to form the channel-active dimer **2**. In single-channel current measurements, the association leads to an opening of the channel and the dissociation to a closing of the channel (Fig. 1B).

With the aim to study ion-channel clustering in membranes, we turned our attention to compound **3**. In **3**, two molecules of **1** are connected via a succinyl linker (in blue) (Fig. 2A).

A priori, there are two types of channel-active associates possible for compound **3** in a membrane (Fig. 2B). One is formed by the dimerization of one **gA** part only. We call this type of dimer the *mono-channel*. The other is formed by the dimerization of both **gA** parts. We call this type the *twin-channel*. The mono-channel and the twin-channel are in equilibrium. The mono-channel of **3** should show channel currents of the size found for **1**, while the twin-channel should show channel currents of much larger size. The two observable types of channel formation could make compound **3** a structurally simple model system for channel clustering. Here, we present structural and functional results concerning the ion-channel activity and the mono-channel/twin-channel equilibrium of **3**.

Materials and methods

Synthesis. Chemicals and reagents were purchased from Aldrich, Sigma, Fluka, and Bachem and used without further purifications. Solvents were purified by distillation. Compounds **1** and **3** were

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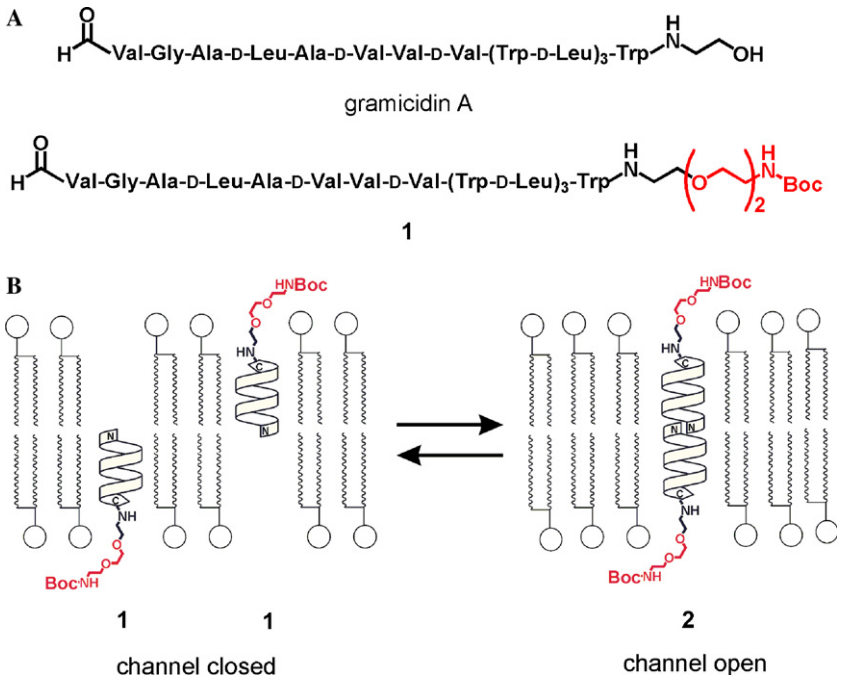

Fig. 1. (A) Structural formula of gramicidin A and **1**; (B) equilibrium between the two channel inactive monomers **1** and the channel active dimer **2** in the membrane.

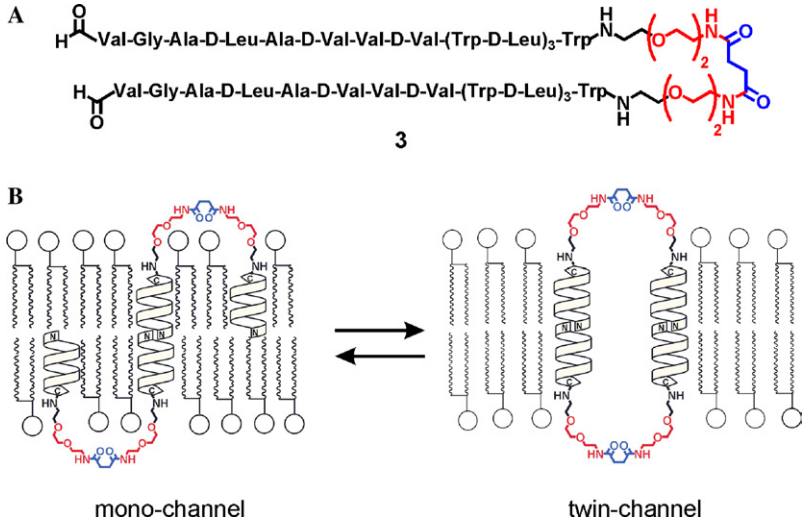

Fig. 2. (A) Structural formula of **3**; (B) equilibrium between the mono-channel and the twin-channel formed by association of two molecules of **3** in the membrane.

synthesized by segment coupling in solution as described [10]. Analytical HPLC was performed with Rainin–Dynamax and Diode Array Detector, prep. HPLC with Rainin–Dynamax/SD1 and UV-Detector.

Ion channel activity. Planar lipid membranes were prepared by painting a solution of the lipid in *n*-decane (25 mg/ml) over the aperture of a polystyrene cuvette with a diameter of 0.15 mm [11]. All experiments were performed at ambient temperature. In the case of DMPC (14:1) 20% cholesterol was used as an additive to stabilize the bilayer. The cation solution at a concentration of 1 M was unbuffered. **gA**, compounds **1** and **3** were dissolved in methanol and added to both sides of the cuvette in homodimer single-channel measurements (end concentration in the cuvette 10^{-11} mol/L), while in the heterodimer measurements, **gA** was added to one side and **3** to the other side. Current detection and recording were performed with a patch-clamp amplifier Axopatch 200B, a Digidata A/D converter, and pClamp6 software (Axon Instruments, Foster City, MA). The acquisition frequency was 5 kHz. The data were filtered with a digital filter at 50 Hz for further analysis.

Circular dichroism spectra. CD-spectra were recorded with Jasco-810 spectrometer. For the preparation of DMPC-micelles, the peptide (**gA** and **3)** and DMPC were dissolved in TFE in a bear-shaped flask and sonicated at 50 °C for 30 min to obtain a homogeneous solution. The solvent was removed in vacuo to produce a thin film in the flask. Water was added and the mixture was sonicated at 50 °C for 30 min.

The clear micellar solution thus prepared should be used on the same day for CD-measurements.

Mass spectroscopy. Mass spectra were recorded with Applied Biosystems Q-Star under ESI-TOF conditions.

NMR. The NMR-spectroscopy data were recorded with Bruker instruments ARX-200, ARX-300, DRX-400, and DRX-500 spectrometers.

Results and discussion

Synthesis

The synthesis of **1** made use of our segment coupling strategy developed for the synthesis of **gA** derivatives [10]. Compound **3** was assembled as shown in Fig. 3A. The Boc-protecting group of **1** was cleaved producing the ammonium salt **4**, which was treated with succinic anhydride and pyridine to produce **5**. A HOAT/HATU coupling of **4** and **5** gave the target compound **3**.

Compounds **1** and **3** were purified by gel filtration (10 g Sephadex LH-20, $CHCl_3$/MeOH 1:1) followed by HPLC (Fig. 3B) and characterized by ^1H NMR and ESI-MS (Supplementary data).

CD-measurements

The CD-spectrum of **3** in DMPC-micelles as lipid-environment indicates a gramicidin-like formation of a typical right-handed-β-helix. Compound **3** exhibits two maxima at 339 nm with and 220 nm typical for a right-handed single stranded β-helix (Fig. 4).

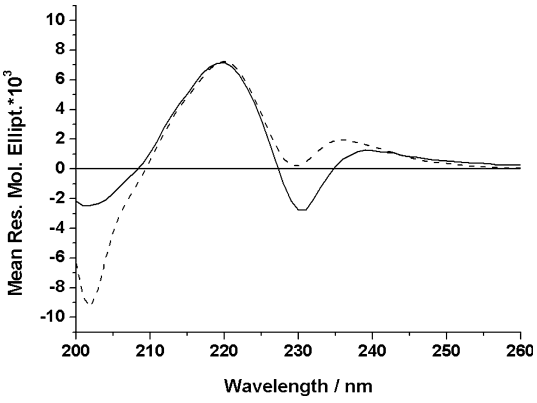

Fig. 4. CD-spectra of compound **3** (solid) and **gA** (dashed) in DMPC-micelles.

Single-channel current measurements

Single-channel measurements for Cs^+ transport in diphytanoylphosphatidylcholine (DPhPC) planar lipid bilayers showed for **1** single-channel events of the size of **gA** (Fig. 5, left). These channels result from the formation of the channel-active dimer **2** through dimerization of two molecules of **1**. In contrast, compound **3** exhibits single-channel events with nearly two times the size of **1**. The double-transport capacity of **3** indicates the presence of the twin-channel in the DPhPC bilayer. Very rarely, mono-channels of half the size of the twin-channels were observed in DPhPC. These results show that the mono-channel/twin-channel equilibrium (Fig. 2B) is shifted strongly towards the twin-channel

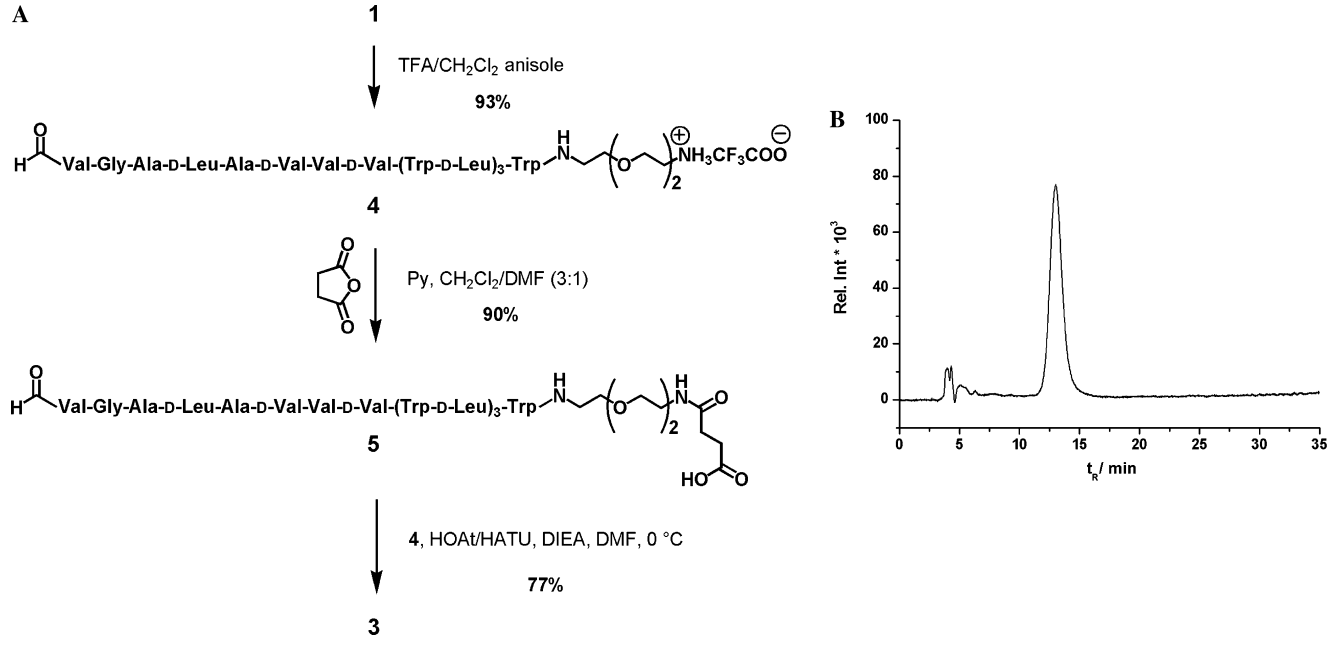

Fig. 3. (A) Synthetic route to **3**; (B) HPLC chromatogram of **3**. RP-C8, I: H_2O, II: CH_3CN/*i*-PrOH 2:1, 80% → 100% II in 30 min, flow rate of 0.7 ml/min, $T = 50$ °C, and $\lambda = 280$ nm.

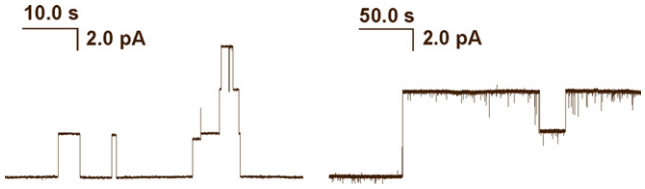

Fig. 5. Single channel trace of **1** (left) **3** (right) in DPhPC and 1 M CsCl at 100 mV.

in DPhPC. The double number of intermolecular hydrogen bonds between the monomers favors the formation of the twin-channel form, while steric strain in the linker region may disfavor it.

Table 1 provides a comparison of the ion-channel properties of **3** with those of **1** and **gA.** The twin-channels from **3** have two times the size of the channels from **1** for K^+ and Na^+, while in case of Cs^+ **3** exceeds the channels of **1** by 75% only. The relative ion permeabilities follow an Eisenman I sequence typical for **gA** [4].

It is instructive to compare these results with the work of Koeppe who studied the ion-channel behavior of **gA** derivatives which were covalently linked at the C-terminus via a peptide chain of different length [12]. A long 23-residue peptide linker was necessary to observe channels of the twin type in this case. Shorter linker led to the formation of mono-channels only. Our results show that twin-channel formation occurs nearly exclusively in DPhPC when short linkers of the flexible ethylene glycol type in **3** are used.

The hydrophobic coupling (match/mismatch) between the membrane and the channel can lead to functional consequences. Examples for a dependence of the channel function on the thickness of the phospholipid bilayer are known for **gA** and its derivatives [12–15]. A hydrophobic mismatch results in shorter dwell times [13]. The channel active **gA** dimer has a hydrophobic length of 22 Å. In order to study the hydrophobic coupling of the mono-channel/twin-channel equilibrium for **3**, single-channel measurements were carried out in dimyristoleoylphosphatidylcholine (DMPC 14:1, Fig. 6A), dipalmitoleoylphosphatidylcholine (DPPC 16:1, Fig. 6B), and dioleoylphosphatidylcholine (DOPC 18:1, Fig. 6B). The channel characteristics for **1** were recorded for comparison reasons.

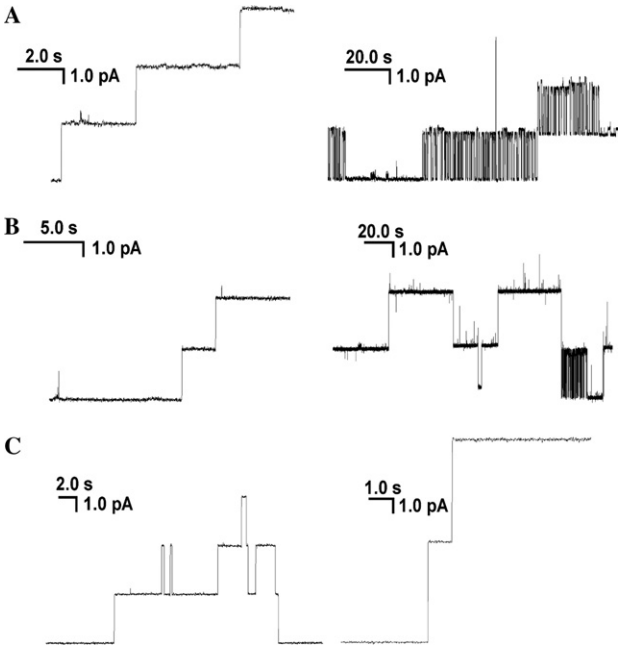

Fig. 6. Current traces for of **1** (left) and **3** (right) with 1 M CsCl at 100 mV in membranes of different thickness (A) DMPC 14:1; (B) DPPC 16:1; and (C) DOPC 18:1.

The effect of the membrane thickness on the mono-channel/twin-channel equilibrium is remarkable. Compound **3** forms mono-channels in the thinner DMPC bilayer but twin-channels in the thicker DOPC bilayer. In DPPC both channel types occur. Amplitude histograms of the single channel events underline this observation (Fig. 7).

The channel active **gA** dimer has a hydrophobic length of 22 Å. The hydrophobic thickness of DOPC (18:1) can be estimated to be 27 Å and that of DMPC (14:1) to be 22 Å [13]. Thus, DOPC (18:1) is too thick for the **gA** dimer. The twin-channel can withstand this hydrophobic mismatch better than the mono-channel, which may explain the dominant formation of the twin-channel in DOPC (18:1). In the case of DMPC (14:1) one expects a good hydrophobic match between the channel and the membrane. Now the mono-channel/twin-channel equilibrium is shifted towards the mono-channel. The driving force for this shift could be

Table 1
Ion channel characteristics of **gA**, **1**, and **3**

Compound	State of conductance Λ/pS			Relative permeability			Dwell time τ/s
	Cs^+	K^+	Na^+	Cs^+	K^+	Na^+	
gA	43.6	26.0	14.8	4.83	2.36	1.00	1.4
1	40.0	20.0	10.2	3.80	2.52	1.00	5.0
3	71.1	40.9	20.9	3.46	2.35	1.00	Several min

States of conductance (Λ/pS), and relative permeabilities of compounds **gA** in asolectine, **1**, and **3** in DPhPC and 1 M MCl electrolytes. Dwell times were determined in asolectine for **gA** and in DPhPC for **1** and **3**, and 1 M CsCl at 100 mV.

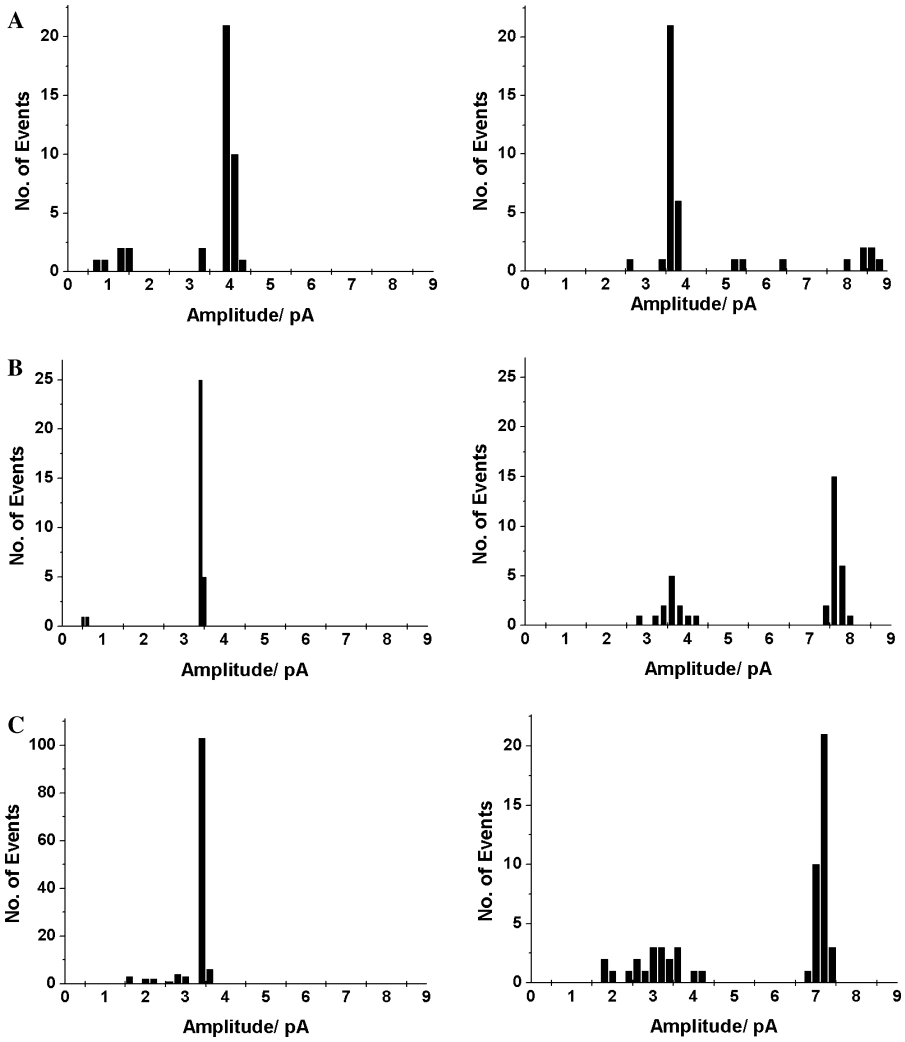

Fig. 7. Single-channel amplitude histograms for **1** (left) and **3** (right) with 1 M CsCl at 100 mV in membranes of different thickness (A) DMPC 14:1; (B) DPPC 16:1; and (C) DOPC 18:1.

steric strain linker region, which in the absence of hydrophobic coupling favors the mono-channel.

Asolectine is a naturally occurring phospholipid mixture, whose stiffness can be increased by addition of cholesterol [16]. Compound **3** shows in asolectine mono-channel behavior (Fig. 8A). In contrast, **3** exhibits twin-channels in asolectine/20% cholesterol (Fig. 8B). Addition of 20% cholesterol to asolectine is enough to increase the membrane stiffness and to stabilize the twin-channel mode.

Compound **3** was studied with **gA** in heterodimer single-channel experiments. **3** was injected to one side of the membrane (10^{-11} mol/L) and **gA** to the other side (10^{-9} mol/L). DPhPC was used as a lipid. The flip-flop in DPhPC did not occur in the time frame of the single-channel experiments (30 min). It was expected to

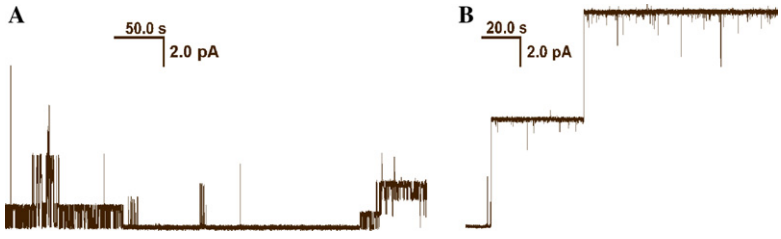

Fig. 8. Single-channel measurements of **3** in asolectine (A) and in asolectine/20% cholesterol (B) for1 M CsCl at 100 mV.

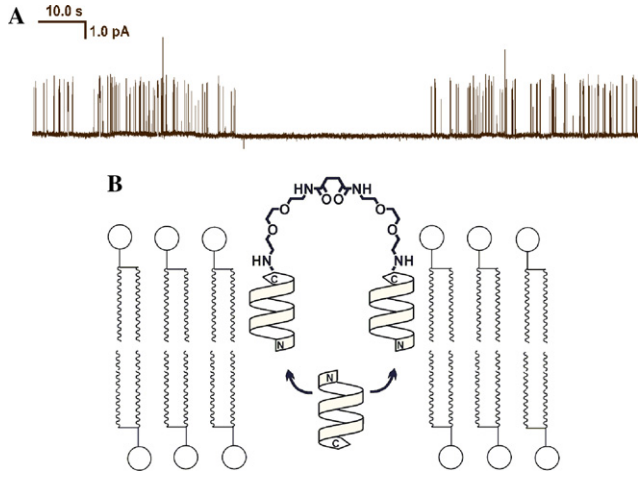

Fig. 9. (A) Current trace of the heterodimer **3** and **gA** in DPhPC and 1 M CsCl at 100 mV; (B) schematic drawing of the heterodimer of **3** and **gA** in the bilayer membrane.

detect two types of current transitions; one should be twin-channel-like transition amplitude and the other should provide **gA**-like transition amplitude. Surprisingly, just flicker channels were detected (Fig. 9A). The concentration of **gA** was increased to 10^{-6} mol/L but no twin-channel-like amplitudes could be detected.

No associate of long lifetime was observed in the heterodimer analysis. This result could be interpreted as a result of a fast exchange of **gA** monomer between both β-helices of the compound **3** (Fig. 9B), which yields these flicker channels.

Conclusion

In conclusion, a novel polyether linked gramicidin derivative **3** was prepared and its mono-channel/twin-channel equilibrium was studied in different phospholipid bilayers. In thin DMPC membranes the mono-channel is the main channel observed. Thicker DOPC bilayers and stiffer membranes favor the twin-channel. These results recommend compound **3** as a simple model system for further studies on the membrane dependence of channel clustering.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.12.170.

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